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Salicylic Acid-Involved in Lead Tolerance Associated with the Maintenance of Reducing Conditions in Mice

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Abstract Lead (Pb^{2+}) intoxication may initiate many disorders in human and animals. In a previous study, we demonstrate that application of salicylic acid protects nuclear DNA integrity against Pb^{2+} stress in mice. In this study, further evidence showed that the exogenous salicylic acid-involved in Pb^{2+} tolerance in mice was associated with the maintenance of reducing conditions in cells, as revealed by an increased antioxidative enzyme activity, elevated reduced glutathione content and ratio to oxidised form of glutathione, and decreased lipid peroxidation. In addition, the presence of a 30 kD protein was tightly linked to the Pb^{2+} detoxification.

Keywords Mice · Salicylic acid · Lead · Antioxidant defense

Lead (Pb) is one of the major hazards for human and animals due to its high toxicity, wide distribution, and persistence in the environment (Adonaylo and Oteiza 1999; Ahamed and Siddiqui Mohd 2007). Increasing evidence indicates that Pb^{2+} exposure may initiate many disorders (for a review see Patrick 2006). Although the biochemical and molecular mechanisms of Pb^{2+} toxicity remain to be elucidated, growing evidence demonstrates that the toxicity of Pb^{2+} is attributed to its oxidative damage (Aykin-Burns et al. 2003; Patrick 2006 and references here; Sandhir and Gill 1995). Higher organisms, however, have evolved a complex of antioxidant defense system comprising low-molecular components such as ascorbate and glutathione, and enzymatic components such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidases (APX), which are involved in the detoxification of reactive oxygen species such as O_2^-, H_2O_2 and $OH^·$ radicals (Valko et al. 2007).

Salicylic acid (SA) is found in significant quantities in a plant-based diet. Humans and animals obtain SA mainly from daily foods, vegetables and fruits. Otherwise, taken orally, aspirin (acetylsalicylic acid), an anti-inflammatory and analgesic drug, is rapidly hydrolyzed to SA in the liver and blood, where it is tightly bound to plasma proteins and distributed to all tissues in the body (Drew et al. 2005). Increasing evidence demonstrates that applied SA can counteract oxidative damage induced by adverse conditions in animals (Dinis-Oliveira et al. 2007; Guerrero et al. 2004). However, the functional mechanism of SA in protecting animals against oxidative damage remains to be further evaluated. In a previous study, we demonstrate that application of SA can protect mice against Pb^{2+}-induced oxidative damage and maintain the nuclear DNA integrity (Li et al. 2007). We further show here that the exogenous SA-improved Pb^{2+} tolerance in mice may be associated with the maintenance of reducing conditions in cells.

Materials and Methods

Five week-old healthy 'Kunming' male albino mice weighing 20 ± 2 g were used in the present experiment. The animals were randomly divided into four groups: normal control group, control + SA treated group, Pb^{2+}-treated group, and Pb^{2+} + SA treated groups, and kept to a cage (20 × 20 × 15 cm) containing five animals with free

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access to food and water under a 12 h light/12 h dark cycle in humidity (60 ± 10%) and temperature (25 ± 2°C) controlled rooms. For Pb^{2+} exposure, the mice were treated by oral gavage of 15 mg Pb^{2+}/kg body weight as lead acetate solution once daily for 7 days. For the Pb^{2+} + SA groups, a mixed solution of lead acetate and 0.5 mM L^{-1} of SA were administered by oral gavage. As a control, equivalent amounts of physiological saline were given. The mouse liver was collected on day 7 of Pb^{2+} exposure and washed three times with ice-cold physiological saline, weighed, wrapped with aluminum foil and stored in liquid nitrogen before use.

The relative body weight increment of mice was calculated using the formula:

$$\frac{(W_1 - W_0)}{W_0} \times 100\%$$

W_0, the mean body weight at the beginning of Pb^{2+} exposure; W_1, the mean body weight at the end of the Pb^{2+} exposure.

Taken 0.5 g tissue, ground in liquid nitrogen with pestle/mortar, added the powder to test tube containing 5 mL ice-cold extract solution consisting of 50 mM phosphate buffer, pH 7.0, 1 mM dithiothreitol, mixed well and incubated for 15 min on ice. The homogenate was centrifuged at 12,000 × *g* for 15 min, and the supernatant was collected to be used to determine the following indices. SOD (EC 1.15.1.1) activity was assayed using the method of Sun et al. (1988). The final volume of the reaction system was 3.0 mL containing 0.1 mM of xanthine, 0.1 mM EDTA, 50 mg L^{-1} bovine serum albumin, 25 mM nitroblue tetrazolium (NBT), 9.9 nM xanthine oxidase, and 40 mM Na_2CO_3 (pH 10.2). The production of formazan was determined at 560 nm in a spectrophotometer at 25°C. One unit of SOD is defined as the amount of enzyme necessary to inhibit the rate of NBT reduction by 50%. CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi (1983). The level of lipid peroxidation was determined by measuring the level of thiobarbituric acid reactive substance (TBARS) following Esterbauer and Cheeseman (1990). The samples were mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid (TBA) 0.67% and were then heated in a boiling water bath for 15 min. TBARS were determined by the absorbance at 535 nm and expressed as nM of malondialdehyde (MDA) formed. Protein concentration was estimated by the method of Lowry et al. (1951) using bovine albumin as standard.

The reduced glutathione (GSH) content was determined as described by Griffith and Meister (1979). The assay was based on sequential oxidation of glutathione by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduction by NADPH in the presence of glutathione reductase (GR). A half gram of fresh material was ground under liquid nitrogen and the powder was added to a test tube containing 5 mL of 2% metaphosphoric acid, mixed well and incubated for 10 min on ice, centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was neutralized by adding 0.6 mL 10% sodium citrate. The oxidised glutathione (GSSG) content was calculated from the difference between total glutathione from DTT-treated samples and GSH from non-DTT-treated samples. For polyacrylamide gel electrophoresis (PAGE) of total liver proteins, the soluble protein extracts prepared for the measurement of the antioxidative enzymes were separated by PAGE using 13% (w/v) acrylamide gels. The proteins were visualized by Coomassie staining (20% ethanol, 5% acetic acid and 0.07% Coomassie Brilliant Blue R250).

All data were obtained from at least three separate experiments and expressed as mean ± standard deviation, and analyzed with one-way ANOVA. The differences were considered significant at $p < 0.05$.

Results and Discussion

Figure 1a shows the relative body weight increment of mice. The Pb^{2+} exposure caused markedly the growth retardation in mice, and application of 0.5 mM L^{-1} SA (corresponding to 2.75 mg SA kg^{-1} body weight) counteracted effectively the Pb^{2+}-induced growth inhibition. This dose of SA had no effect on the growth of the non-Pb^{2+} stressed mice (Fig. 1a), but higher SA inhibited mouse growth (data not shown). This is also true in *planta* where application of physiological levels of SA (mM concentrations) confers plant tolerance to various detrimental environments (for a review see Yuan and Lin 2008), but the SA high-accumulation in plants inhibits the plant growth (Bowling et al. 1997; Li et al. 2001).

The applied SA partially alleviated the Pb^{2+}-induced inhibition of SOD and CAT activities, in which the SOD activity in Pb^{2+} + SA group was comparable to that in control (Fig. 1b), and the CAT activity was also significantly higher than that in the Pb^{2+}-treated group (Fig. 1c). Although no visible influence on the body weight increment, the application of SA altered the activity of SOD and CAT in the non-stressed mice, with SOD activity being increased and CAT decreased compared to their controls, respectively (Fig. 1b, c).

The Pb^{2+} exposure decreased the levels of GSH (Fig. 1d) and ratio of GSH to GSSG (Fig. 1e). The concomitant administration of SA totally eliminated the Pb^{2+}-caused reduction of the levels of GSH and ratio of GSH to GSSG. In addition, the SA treatment also elevated the GSH levels and ratio to GSSG in unstressed animals (Fig. 1d, e). Under the Pb^{2+} exposure, the MDA levels were significantly

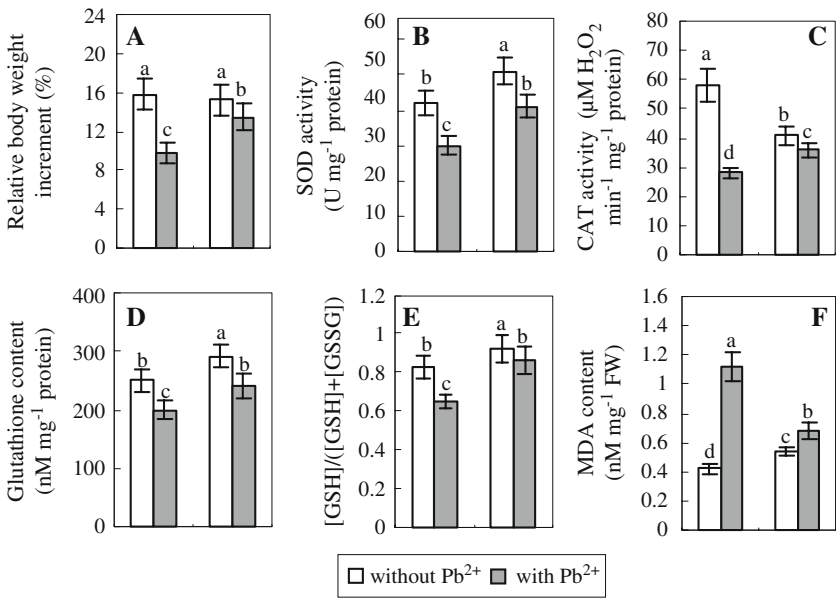

Fig. 1 Effect of Pb^{2+} exposure on relative body weight increment (**a**), superoxide dismutase (**b**), catalase (**c**), glutathione (**d**), the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) (**e**), and malondialdehyde (MDA) content (**f**) in mice. Left group of columns: control; Right group of columns: oral gavage of 15 mg Pb^{2+} kg^{-1} body weight as lead acetate solution once daily for 7 days. The different letters (a, b, etc.) indicate a significant difference at $p < 0.05$

increased, and the application of SA counteracted efficiently the Pb^{2+}-caused lipid peroxidation, even though the SA also induced the MDA accumulation in non-stressed mice (Fig. 1f).

Figure 2 clearly showed that a 30 kD protein was always linked to the Pb^{2+} detoxification. The Pb^{2+} exposure caused the protein disappearance, and the supplementation of SA maintained the protein unchanged, with the SA-induced increase of the antioxidant capacity (Fig. 1b–e) and decrease of the lipid peroxidation (Fig. 1f), suggesting that the presence of the 30 kD protein may be correlated to the reducing conditions in cells. To address this issue, we substituted the SA in the SA + Pb^{2+} group with 100 mg kg^{-1} vitamin C. Vitamin C is a well known free-radical scavenger and has been shown to inhibit lipid peroxidation in lead-exposed animals (Patra et al. 2001). The result showed that the application of vitamin C was also tightly linked to the presence of the 30 kD protein (Fig. 2). These data suggest that the 30 kD liver protein may be considered as an additional parameter in assessing ecological risk, and for protective drug screening and selection.

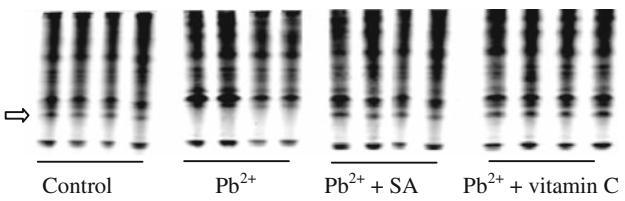

Fig. 2 Polyacrylamide gel electrophoresis of total proteins in mouse livers. *Arrow* indicates a protein with a relative molecular weight of approximately 30 kD. The samples in each group were taken from four individual animals, respectively

The determination of antioxidative capacity and lipid peroxidation level in animals has been used extensively for monitoring the toxicity of various adverse stresses. The present investigation shows that the Pb^{2+} exposure (at 15 mg kg^{-1} body weight of Pb^{2+}) caused oxidative stress in mouse liver as revealed by significantly elevated TBARS levels (Fig. 1f), which is consistent with other observations in other animal models (Villeda-Hernandez et al. 2001; Hsu and Guo 2002; Ahamed and Siddiqui Mohd 2007; Berrahal et al. 2007; Massó et al. 2007). The SOD and CAT activities show that the Pb^{2+} exposure caused decreases of the two enzyme activities, suggesting that the heavy metal caused the lipid peroxidation through a regulation of the antioxidative status. This is in agreement with many other reports (Adonaylo and Oteiza 1999; Bennet et al. 2007; Berrahal et al. 2007). Concomitant administration of SA partially alleviated or completely removed the Pb^{2+}-caused enzyme activity inhibition (Fig. 1b, c), accompanied by a restoration in the animal weight increment (Fig. 1a) and a reduction of TBARS level caused by Pb^{2+} exposure (Fig. 1f). Glutathione has been shown to be a significant factor in heavy metal detoxification where in addition to acting as an important antioxidant for quenching free radicals, glutathione also directly binds to toxic metals, preventing them from binding to cellular proteins and causing damage to both enzymes and tissue (Patrick 2002, 2006 and references here). Glutathione exists in both a reduced and oxidised form (glutathione disulphide), and its influence on cellular redox status depends on both the GSH/GSSG ratio and the concentration of GSH (Schaffer and Buettner 2001). The data in this study showed that the concomitant administration of SA totally eliminated the Pb^{2+}-caused reduction of the level of

GSH and ratio of GSH to GSSG. In addition, the SA treatment also elevated the GSH levels and ratio to GSSG in unstressed animals (Fig. 1d, e). These data suggested that the SA-improved Pb^{2+} tolerance in mice may be correlated to the maintenance of reduced glutathione levels and ratio to oxidised form of glutathione, as well as increased antioxidative enzyme activity, resulting in reducing conditions in cells. To our knowledge, there is little parallel evidence in animals. In *planta*, however, unarguable evidence has demonstrated that SA levels and signaling are necessary to establish systemic acquired resistance (Gaffney et al. 1993), and the mechanism is tightly linked to the maintenance of the cellular reducing conditions (Mou et al. 2003; Tada et al. 2008).

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References

Adonaylo VN, Oteiza PI (1999) Lead intoxication: antioxidant defenses and oxidative damage in rat brain. Toxicology 15:77–85. doi:10.1016/S0300-483X(99)00051-7

Aebi HE (1983) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis, vol III, 3rd edn. Verlag Chemie, Weinheim, pp 273–286

Ahamed M, Siddiqui Mohd KJ (2007) Environmental lead toxicity and nutritional factors. Clin Nutr 26:400–408. doi:10.1016/j.clnu.2007.03.010

Aykin-Burns N, Laegeler A, Ellog G, Ercal N (2003) Oxidative effects of lead in young and adult fisher 344 rats. Arch Environ Contam Toxicol 44:417–420. doi:10.1007/s00244-002-2023-4

Bennet C, Bettaiya R, Rajanna S, Baker L, Yallapragada P, Brice JJ, White SL, Bokara KK (2007) Region specific increase in the antioxidant enzymes and lipid peroxidation products in the brain of rats exposed to lead. Free Radic Res 41:267–273. doi:10.1080/10715760600889855

Berrahal AA, Nehdi A, Hajjaji N, Gharbi N, El-Fazâa S (2007) Antioxidant enzymes activities and bilirubin level in adult rat treated with lead. C R Biol 330:581–588. doi:10.1016/j.crvi.2007.05.007

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 9:1573–1584

Dinis-Oliveira RJ, Remião F, Duarte JA, Navarro AS, Bastos ML, Carvalho F (2007) Full survival of paraquat-exposed rats after treatment with sodium salicylate. Free Radic Biol Med 42:1017–1028. doi:10.1016/j.freeradbiomed.2006.12.031

Drew JE, Arthur JR, Farquharson AJ, Russell WR, Morrice PC, Duthie GG (2005) Salicylic acid modulates oxidative stress and glutathione peroxidase activity in the rat colon. Biochem Pharmacol 70:888–893. doi:10.1016/j.bcp.2005.06.011

Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 186:407–421. doi:10.1016/0076-6879(90)86134-H

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754–756. doi:10.1126/science.261.5122.754

Griffith OW, Meister A (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (s-n-butylhomocysteine sulfoximine). J Biol Chem 254:7558–7560

Guerrero A, González-Correa JA, Arrebola MM, Muñoz-Marín J, Sánchez de la Cuesta F, de la Cruz JP (2004) Antioxidant effects of a single dose of acetylsalicylic acid and salicylic acid in rat brain slices subjected to oxygen-glucose deprivation in relation with its antiplatelet effect. Neurosci Lett 358:153–156. doi:10.1016/j.neulet.2004.01.036

Hsu PC, Guo YL (2002) Antioxidant nutrients and lead toxicity. Toxicology 180:33–44. doi:10.1016/S0300-483X(02)00380-3

Li X, Clarke JD, Zhang Y, Dong X (2001) Activation of an EDS1-mediated *R*-gene pathway in the *snc1* mutant leads to constitutive, NPR1-independent pathogen resistance. Mol Plant Microbe Interact 14:1131–1139. doi:10.1094/MPMI.2001.14.10.1131

Li TT, Li RG, Hao L, Xu X, Na J (2007) Exogenous salicylic acid regulation of lead-induced oxidative stress in mice. Acta Sci Circum 27:802–806

Lowry OH, Rosenbrough NJ, Far AL, Randel RJ (1951) Protein measurement with folin–phenol reagent. J Biol Chem 193:265–275

Massó EL, Corredor L, Antonio MT (2007) Oxidative damage in liver after perinatal intoxication with lead and/or cadmium. J Trace Elem Med Biol 21:210–216. doi:10.1016/j.jtemb.2007.03.002

Mou ZL, Fan WH, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113:935–944. doi:10.1016/S0092-8674(03)00429-X

Patra RC, Swarup D, Dwidedi SK (2001) Antioxidant effects of atocopherol, ascorbic acid and L-methionine on lead-induced oxidative stress of the liver, kidney and brain in rats. Toxicology 162:81–88. doi:10.1016/S0300-483X(01)00345-6

Patrick L (2002) Mercury toxicity and antioxidants: part I: role of glutathione and alpha-lipoic acid in the treatment of mercury toxicity. Altern Med Rev 7:456–471

Patrick L (2006) Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. Altern Med Rev 11:114–127

Sandhir R, Gill KD (1995) Effect of lead on lipid peroxidation in liver of rats. Biol Trace Elem Res 48:91–97. doi:10.1007/BF02789081

Schaffer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–1212. doi:10.1016/S0891-5849(01)00480-4

Sun Y, Oberley LW, Li YA (1988) A simple method for clinical assay of superoxide dismutase. Clin Chem 34:497–500

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X (2008) Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. Science 321:952–956. doi:10.1126/science.1156970

Valko M, Leibfritz D, Moncola J, Cronin MTD, Mazura M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Inter J Biochem Cell Biol 39:44–84. doi:10.1016/j.biocel.2006.07.001

Villeda-Hernandez J, Barroso R, Mendez M, Nava C, Huerta R, Rios C (2001) Enhanced brain regional lipid peroxidation in developing rats exposed to low level lead acetate. Brain Res Bull 55:247–251. doi:10.1016/S0361-9230(01)00512-3

Yuan S, Lin HH (2008) Role of salicylic acid in plant abiotic stress. Z Naturforsch (C) 63:313–320